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Technical Bulletin

CompoZr® Custom Zinc Finger Nuclease (ZFN)

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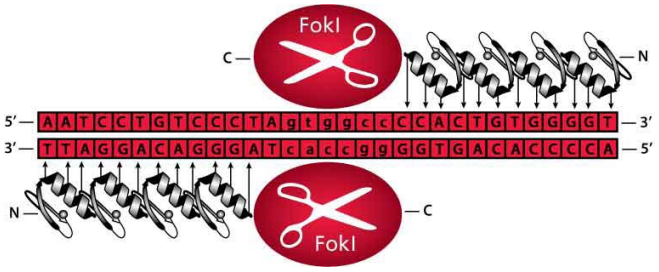
Product Description

CompoZr Zinc Finger Nucleases (ZFNs), a class of engineered DNA-binding proteins, facilitate targeted genome editing by binding to a user-specified locus and causing a double-strand break (DSB). The cell then employs endogenous DNA repair processes, either non-homologous end joining (NHEJ) or homology-directed repair (HDR), to heal this targeted DSB. These repair processes can be channeled to generate precisely targeted genomic edits resulting in an organism or cell lines with specific gene disruptions (knockouts), integrations, or modifications.



CompoZr ZFNs consist of two functional domains: a DNA-binding domain comprised of a chain of zinc finger proteins and a DNA-cleaving domain comprised of the nuclease domain of F*okI*. Each zinc finger DNA binding protein recognizes a 3 base pair target. By combining 4-6 zinc finger proteins together, each ZFN can target and specifically bind a 12-18 base pair sequence. Importantly, the endonuclease domain of F*okI* has been reengineered to function as an obligate heterodimer in order to cleave DNA (Miller et al., 2007). This means a *pair* of ZFNs is required to bind and cut the genomic DNA at the targeted site and this property is used to ensure specificity. The target sequences for each ZFN must be separated by 5-7 base pairs to allow formation of the catalytically active F*okI* dimer. The 24-36 base pair DNA binding specificity and additional positional constraints drive a very high degree of precision in genome editing. Each set of CompoZr ZFNs has been validated by Sigma to cleave at the genomic site of interest.

Provided in the kit are the ZFNs designed against the user-specified gene in the target organism. We provide two plasmids encoding the ZFN pair for the best performing ZFN and the corresponding ready-to-deliver mRNA for the same ZFN pair. Included in the kit for ZFNs targeted to *human, rat, mouse and hamster* are primers for screening as well as genomic DNA from samples where the supplied ZFN has been tested. For ZFNs against all other organisms, plasmid DNA for the next two highest efficiency pairs of ZFNs is also provided.

The ZFNs can be delivered to cells as either mRNA or plasmid DNA. The major advantage of mRNA is that it eliminates the need to use different promoters for ZFN expression in different cell types, as mRNA is universal to all cell types. For instance, mRNA tends to work better in HeLa cells than plasmid since the CMV promoter on ZFN plasmids is not as active in HeLa as in other common cell types. Also, it eliminates the necessity of nuclear delivery because mRNA is translated into protein in the cytoplasm whereas DNA expression vectors have to enter the nucleus to be transcribed. *Please make sure the utmost caution is taken when handling mRNA to avoid degradation and loss of activity.* There are advantages to using plasmid DNA as well. ZFN expression is prolonged when originating from plasmid DNA, and may help in cell types where lower frequencies of ZFN cutting are observed. Additionally, plasmid DNA provides a source for generating more mRNA when existing mRNA stocks are used up.

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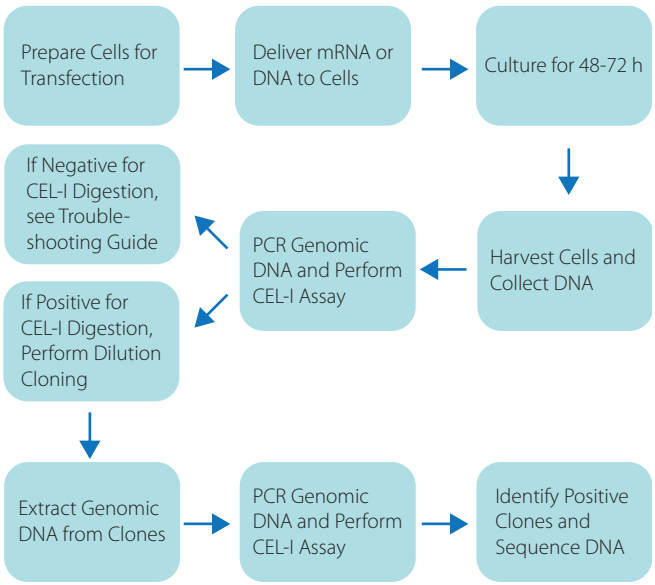
Cell line modification using ZFNs is simple and relies on standard processes such as transfection, dilution cloning, and genotyping. We have successfully used CompoZr ZFNs for targeted gene editing in a variety of cell types including K562, HEK293, HeLa, A549, MCF-7, C6, Neuro-2A, LNCaP and CHO cells. The CompoZr technology is compatible with standard methods of DNA delivery into cells, including microinjection, lipid-based transfection, electroporation, and nucleofection. Following delivery into the cell, ZFN-mediated editing will occur in as little as three days, followed by dilution cloning and screening of individual clones. The targeted deletion and integration events that happen in ZFN-treated cells give rise to a population of cells containing biallelic or monoallelic modification, or unmodified gene sequence. From this pool, it is possible to rapidly isolate several cell lines containing either heterozygous or homozygous mutations. Aneuploid cell lines are also conveniently modified since triploid to hexaploid loci have all been successfully modified using ZFNs in the absence of antibiotic selection.

Previous methods for generating modified cell lines have relied heavily on random integration of a plasmid construct and required extensive screening approaches to generate a single, usable cell line. Much of the work with targeted genome modifications has been limited to mouse models and still requires several rounds of reproduction to generate progeny for experiments. The limited applicability of prevalent techniques prohibits numerous laboratories from attacking important biological questions. CompoZr ZFNs provide a new and exciting way to target many organisms and cell types for modification at a single defined locus, allowing mutations, correction, and deletions within the natural chromosomal context. In addition, the high efficiency of gene modification using CompoZr ZFNs greatly reduces the time it takes to generate a clonal cell line for research use.

Welcome to the future of genetics!

Procedure Overview



Precautions and Disclaimer

The CompoZr Zinc Finger Nuclease Kit is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage and Stability

Store the kit at –80 °C immediately upon arrival. When removing kit components, ensure the tubes containing mRNA, which will not be immediately used, remain frozen. Please avoid repeated freeze thawing of the pure mRNA component and remove only as many vials from the kit for thawing as are needed for immediate experiments. All components can be stored at –80 °C for up to 12 months.

Practice aseptic techniques to avoid RNase contamination of the components. Caution must be taken to avoid RNase contamination during ZFN mRNA preparation, especially during the RNA elution and post-elution handling steps. The work area and the pipette set must be free of RNases. Use RNase*ZAP*® to decontaminate the work area and the pipette set if necessary. Use RNase-free pipette tips, preferably those with an aerosol barrier. Always wear gloves and change them often. Keep reagent vials and sample tubes closed when not in use.

Kit Components

Component	Catalog Number	Quantity
ZFN mRNA	MRZFN	1 each
pZFN1	D1ZFN	20 µg
pZFN2	D2ZFN	20 µg
ZFN Primer F	PFZFN	25 µmoles
ZFN Primer R	PRZFN	25 µmoles
ZFN Control DNA	CDZFN	1 vial

Note: Included in the kit for ZFNs targeted to human, rat, mouse and hamster are primers for screening as well as genomic DNA from samples where the supplied ZFN has been tested. For ZFNs against all other organisms, plasmid DNA for the next two highest efficiency pairs of ZFNs is also provided.

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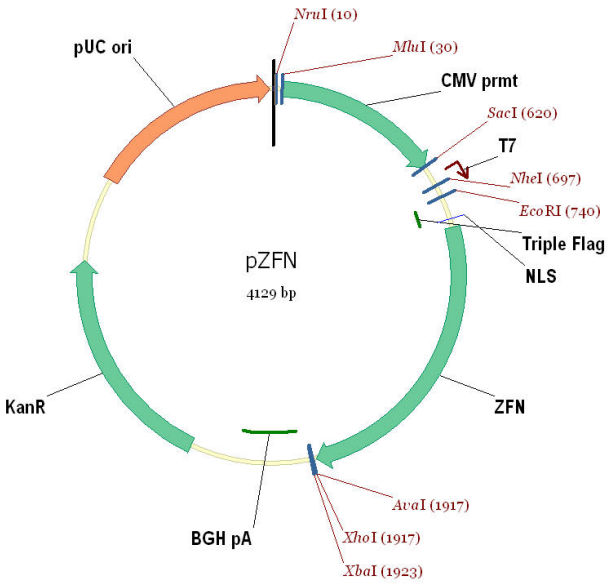

Map of ZFN plasmid

Protocols

Delivery of ZFNs for Gene Knockout

Reagents and Equipment Recommended But Not Provided

Nucleofection® reagents

Electroporation reagents

*Trans*IT®-mRNA Transfection Reagent (Mirus Bio Catalog Number MIR 2225, LLC)

EX-CELL® GTM-3 (Catalog Number G9916) - If using electroporation

Hank's Balanced Salt Solution (HBSS, Catalog Number H6648)

Cell Line Nucleofector® Kit V (Lonza Catalog Number VCA-1003)

a. Nucleofection
Note: The following procedure has been successfully applied to K562 cells. For cells other than K562, please follow cell line-specific instructions which can be found at http://www.lonzabio.com

i. Seed the cells at a density of 2×10^5 cells/ml the day before transfection.
ii. On the day of transfection, take out Cell Line Nucleofector Kit V and let warm to room temperature.
iii. Add the supplement to the Nucleofection Solution V according to the manufacturer's protocol.
iv. Count the cells. Cell density should be between $2.5–5 \times 10^5$ cells/ml.
v. Fill a 6-well plate with 2 ml of medium in each well and prewarm in a CO_2 incubator at 37 °C for at least 20 minutes prior to nucleofection.
vi. Centrifuge 2×10^6 cells per transfection (8×10^6 total) at 200 × *g* for 5 minutes.
vii. Wash cells twice with 20 ml of HBSS.
viii. Prepare experimental tubes:

Reagent \ Tube - Label	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µl (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**Δ	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied. Total volume in the nucleofection should be less then 10 µl.

**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.

ΔOptimal concentration of the ZFN plasmid may need to be determined based on your cell line of interest.

ix. Remove the 6-well plate containing media from step (v) from incubator.
x. Resuspend cells in 400 µl (100 µl/reaction) of Nucleofection Solution V.
Note: Do not leave cells in nucleofection solution longer than 15 minutes as this will greatly diminish transfection efficiency. To minimize exposure time of the mRNA/DNA to the cells and the cells to the cuvette, perform the reactions separately and quickly.
xi. One reaction at a time, add 100 µl of cells to each DNA or mRNA-containing tube. Transfer the mixture to a 2 mm electroporation cuvette and nucleofect on a Nucleofector with program T-016 for K562 cells.
Note: Nucleofection solutions and programs are cell line specific.
xii. Immediately after nucleofection of each sample, use a transfer pipette to add ~500 µl of the prewarmed medium from the 6-well plate in step (ix) to the cuvette. Then, carefully transfer cells from the cuvette to the remaining prewarmed medium in the 6-well plate.
xiii. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

b. Electroporation
Note: The following procedure has been successfully applied to HEK293 cells. Each cell line should be optimized for electroporation conditions using a reporter construct, such as a GFP expressing plasmid.

i. Seed the cells 1–3 days before transfection so the cells are ~80% confluent on the day of transfection.
ii. On the day of transfection, harvest and count cells.
iii. Fill a 6-well plate with 2 ml of medium in each well and prewarm in a CO_2 incubator at 37 °C for at least 20 minutes prior to electroporation.
iv. Centrifuge 2×10^6 cells per transfection (8×10^6 total) at 200 × *g* for 5 minutes

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v. Wash cells twice with 20 ml of EX-CELL GTM-3 (Catalog Number G9916)

vi. Prepare experimental tubes:

Reagent \ Tube - Label	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µl (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied. Total volume in the electroporation should be less then 10 µl.

**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.

vii. Remove the 6-well plate containing media from step (iii) from incubator.

viii. Resuspend cells in 800 µl of EX-CELL GTM-3 medium.

ix. One sample at a time, add 200 µl of cells to each DNA or mRNA-containing tube. Transfer the mix to a 2 mm cuvette and electroporate with the following conditions: voltage: 115 V, capacitance: 950 µF

x. Immediately after electroporation of each sample, use a transfer pipette to add ~500 µl of the prewarmed medium to the cuvette to help transfer the cells to the medium in the 6-well.

xi. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

c. Lipid-based transfection reagents

Note: The following procedure was designed for use with the TransIT-mRNA Transfection Kit. A549, HCT116, HeLa, and HEK293 cells have been successfully transfected using this procedure. Conditions may have to be optimized if other transfection reagents and cell lines are used.

i. Cells should be at 60–90% confluency at the time of transfection. The day before transfection, seed 8×10^5 cells in 2 ml of complete medium in a 6-well plate.

ii. The day of transfection, remove the medium and add 1 ml of fresh complete medium to cells prior to transfection.

iii. Prepare experimental tubes. Mix reagents by pipetting.

Reagent \ Tube - Label	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
TransIT-mRNA Reagent	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Serum-Free Medium	100 µl	100 µl	100 µl	100 µl
mRNA Boost Reagent	1.25 µl	1.25 µl	1.25 µl	1.25 µl

iv. To the appropriate tubes, add the following additional reagents:

Reagent \ Tube - Label	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µl (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied.

**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.

v. Incubate the mixture at room temperature for 2 minutes. **DO NOT** let samples incubate for more than 5 minutes.

vi. Add the entire mix (~103 µl) of *Trans*IT-Boost-Medium-mRNA to the cells dropwise. Gently rock the plate to mix the complexes, do not swirl.

vii. Incubate in a CO_2 incubator at 37 °C.

Harvesting Genomic DNA after Delivery of ZFNs

Reagents and Equipment Recommended But Not Provided

GenElute™ Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70)

Hank's Balanced Salt Solution (HBSS, Catalog Number H6648)

Lysis Solution for Blood (L3289)

Neutralization Solution for Blood (N9784)

AlumaSeal™ II (A3250)

d. 6-well plates

Note: Do NOT harvest ALL of your pooled cells. It is important to maintain the culture in order to have cells to single cell dilution clone after you confirm that you have Cel-I digestion products. We recommend subculturing about 1/5 of the pooled cells, and harvesting genomic DNA from the rest of the population.

i. One to three days after transfection, collect the cells to prepare chromosomal DNA using the GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70).

e. 96-well plates

Note: Prior to harvesting genomic DNA from single cell derived clones in a 96-well plate, you should consolidate clones to reduce the number of plates that will be manipulated. Some wells will have no cells prior to consolidation. After you make your consolidation plate, you should make 3 replica plates. One plate will be used as a glycerol stock, one plate will be used to harvest genomic DNA and perform the CEL-I assay, and one plate will be used as a working plate.

i. Harvest genomic DNA for each well: *Note: The following example is optimized for adherent HCT116 cells and extraction efficiencies may vary for different cell types.*

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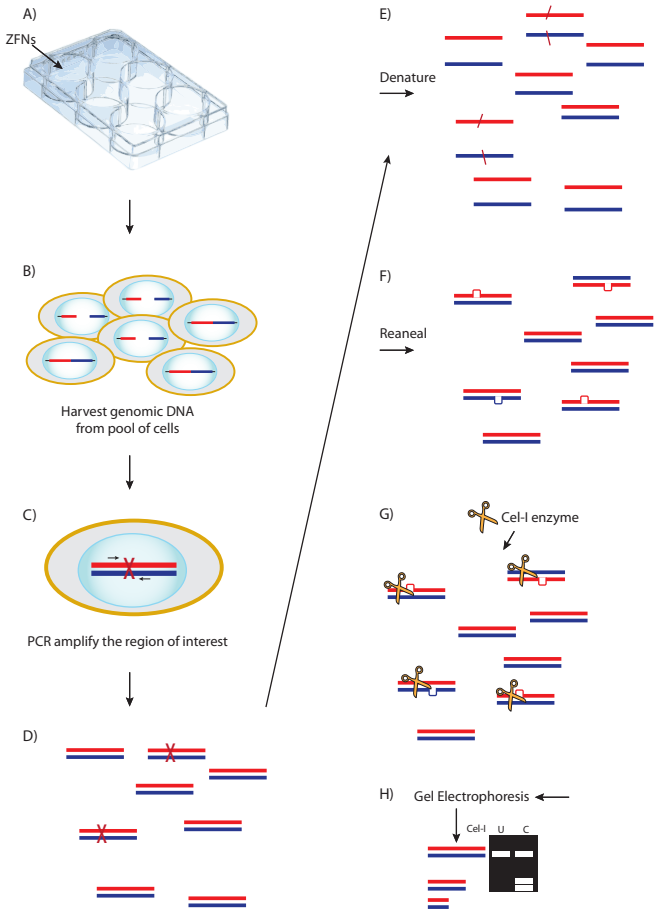
a. Aspirate the medium from cells (cells should be confluent).
b. Wash cells with 100 µl of HBSS (Catalog Number H6648).
c. Add 20 µl of Lysis Solution for Blood (Catalog Number L3289) to each well, then rock back and forth to ensure the entire well is covered.
d. Seal 96-well plate with AlumaSeal II™ (Catalog Number A3250).
e. Incubate at 75 °C for 15 minutes.
f. Add 180 µl of Neutralization Solution for Blood (Catalog Number N9784) and mix by pipetting.
g. DNA can now be used directly for PCR or stored at 4 °C for up to 6 months.

SURVEYOR Mutation Detection Assay (CEL-I Assay)

CEL-I Background

After ZFNs make a double strand break at the target site, the cell uses two main mechanisms to repair the broken chromosome - HDR and NHEJ. NHEJ is more efficient in most cell types and can be an error-prone process that introduces deletions and insertions at the cleavage site. To measure the cleavage efficiency of ZFNs in the cell, we recommend using the CEL-I or SURVEYOR assay, which takes advantage of the NHEJ process. In the assay, the target region is amplified in a PCR reaction using genomic DNA from the transfected cell pool as template. If ZFNs are active, the genomic DNA will be a mixture of wild-type and NHEJ products (insertions or deletions at the target site). The PCR product is then denatured under high temperatures. When the temperature is gradually lowered, some wild-type and NHEJ products hybridize to form double strand DNA with mismatches around the cleavage site, which can be cleaved by an enzyme called CEL-I or SURVEYOR (see figure below).



Schematic of the CEL-I Assay used to detect ZFN activity. (A) ZFN plasmid or mRNA is delivered to cells. (B) Expressed ZFNs bind and cut their target sequence creating a double-strand break (DSB) in a portion of the cells. (C) Aberrant repair of some DSBs by non-homologous end joining (NHEJ) results in insertion, deletion or substitution (depicted by red X). (C, D) Genomic DNA is harvested from the transfected pool of cells and amplified at the locus of interest. (E-F) PCR product is denatured and re-annealed creating heteroduplex formation between wild type and modified amplicons. (G) The CEL-I mismatch endonuclease assay results in cleavage of heteroduplex molecules. (H) CEL-I enzyme digests are resolved by PAGE. The observed ratio of cleavage product to parental band indicates the fraction cut, and hence, efficiency of ZFNs. On top of the black box in (H) representing an electrophoresis gel on the right, lane U stands for a CEL-I-uncleavable sample indicative of no heteroduplex formation, and lane C stands for a CEL-I-cleavable sample indicative of heteroduplex formation and, therefore, ZFN cleavage.

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CEL-I Protocol

Reagents and Equipment

Expand High Fidelity^{PLUS} PCR System (Roche Catalog Number 03 300 242 001)

dNTPs (Sigma Catalog Number D7295)

DirectLoad™ WideRange DNA Ladder (Sigma Catalog Number D7058)

Nuclease S (Cel-I enzyme) + Enhancer (Transgenomic SURVEYOR Kit, Catalog Number 706025)

10% PAGE-TBE gel

Thermocycler

Genomic DNA (ZFN-treated, untreated, and control*)

Validated PCR primers*

*supplied with CSTZFN kits for human, mouse, rat, and Chinese hamster gene targets; gene targets for other species require user-supplied primers and control genomic DNA

a. PCR optimization
Success of the CEL-I assay hinges on the quality of the initial PCR amplification at the locus of interest. We highly recommend optimizing PCR conditions according to the specific polymerase, genomic DNA isolation method, and cycling conditions used by the investigator. ZFN kits for human, mouse, rat and Chinese hamster gene targets include both validated primers and control genomic DNA (ZFN-treated). ZFN kits for other species require primers and control DNA to be supplied by the user. In these cases, the following guidelines may be used as a starting point for primer design and PCR optimization:

- Limit amplicon size to between ~200bp and ~400bp for optimal resolution in PAGE and to reduce background
- Design primers such that the ZFN cut site is located approximately one-third of the total distance from either end of the amplicon (cleavage occurring too close to one end results in a digested fragment not easily seen in the gel)
- Blast primers against genomic sequence to avoid nonspecific amplification
- Use primers with high annealing temperatures in order to achieve specificity of amplification (56-60 °C is suggested)
- Order two or three primers for both forward and reverse annealing and choose the best combination
- Amplification of normal genomic DNA, followed by Cel-I digest, will help you identify primers resulting in the cleanest Cel-I assay (the less smear or banding after digest, the better)

b. Genomic DNA isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70).

i. PCR amplify the genomic DNA from the 4 transfected samples. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Roche Expand High Fidelity^{PLUS} PCR System. Optimization of the conditions may be necessary if another polymerase is used.
Note: In addition to the transfected samples, it is recommended to include a control reaction using the included control genomic DNA (CDZFN).

Reagent	Volume
Water, PCR Reagent	Adjustable
5× PCR buffer	10 µl
dNTPs (10 mM)	1 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µl
ZFN primer F (provided) (25 µM)	1 µl
ZFN primer R (provided) (25 µM)	1 µl
Genomic DNA	200 ng
Total volume	50 µl

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30 cycles
Annealing	50-60 °C*	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

* Check CofA for the specific annealing temperature for your primers

ii. Take 10 µl of PCR reaction from each sample (4 experimental + control) and use the following program on a thermocycler:
95 °C, 10 minutes
95 °C to 85 °C, –2 °C/second
85 °C to 25 °C, –0.1 °C/ second
4 °C, indefinitely
Note: This step can also be performed without a thermocycler. For specific instructions, see the Transgenomic User Guide for the Transgenomic SURVEYOR Mutation Detection Kit for Standard Gel Electrophoresis.

iii. Add 1 µl of enhancer and 1 µl of Nuclease S (from Transgenomic Catalog Number 706025) to each reaction and incubate at 42 °C for 20-40 minutes.
Note: A master mix of enhancer and nuclease S can be made just prior to digest. Do not allow enhancer and nuclease S to incubate together for extended periods prior to digest.

iv. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058) (see figure below).

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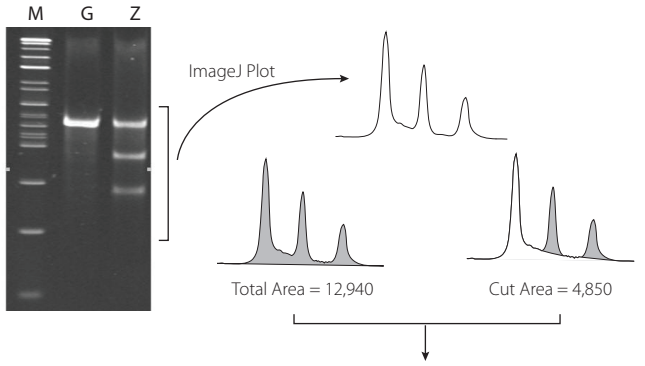
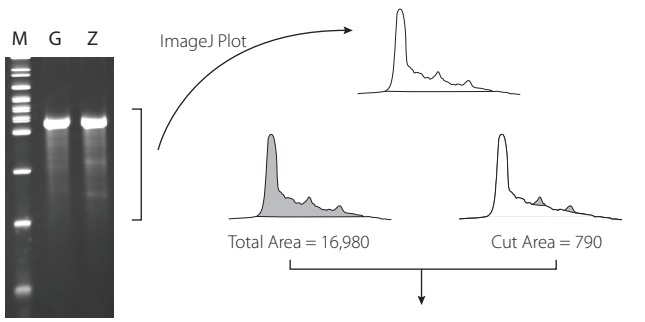


Fraction Cut = 4,850/12,940 = **0.37**
ZFN Activity (a.k.a. % NHEJ) = 100 × (1-(1-**0.37**)$^{0.5}$) = **21%**

Definitions:
Fraction Cut = estimate of frequency of mismatched PCR fragments.
ZFN Activity (a.k.a. % NHEJ) = estimate of mutated alleles in pooled cell population (reported in product Certificate of Analysis).

An example of a highly active ZFN pair in the CEL-I Assay in K562 cells.

Cells were transfected via nucleofection and harvested 1 day after transfection. Genomic DNA was harvested using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70), and PCR and Cel-I digestions were done as described above. Samples were run on a 10% PAGE-TBE gel. M: DirectLoad WideRange DNA Marker (Catalog Number D7058). G: GFP transfected cells Z: Cells transfected with ZFN alone.



Fraction Cut = 790/16,980 = **0.05**
ZFN Activity (a.k.a. % NHEJ) = 100 × (1-(1-**0.05**)$^{0.5}$) = **2.4%**

Definitions:
Fraction Cut = estimate of frequency of mismatched PCR fragments.
ZFN Activity (a.k.a. % NHEJ) = estimate of mutated alleles in pooled cell population (reported in product Certificate of Analysis).

An example of a ZFN pair with lower activity in the CEL-I Assay in K562 cells.

Cells were transfected via nucleofection and harvested 1 day after transfection. Genomic DNA was harvested using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70), and PCR and Cel-I digestions were done as described above. Samples were run on a 10% PAGE-TBE gel. M: DirectLoad WideRange DNA Marker (Catalog Number D7058). G: GFP transfected cells Z: Cells transfected with ZFN alone.

Note: If the CEL-I assay on a pool of cells yields a CEL-I digestion product for your "ZFN alone" sample, you can move on to single cell dilution cloning in order to find a pure clone (derived from a single cell) that has been modified by the ZFNs. After about 3-4 weeks, single cells should have grown into a colony that covers most of a well in a 96-well plate. At this time, you should consolidate clones to reduce the number of plates that will be manipulated. Some wells will have no cells prior to consolidation. After you make your consolidation plate, you should make 3 replica plates. One plate will be used as a glycerol stock, one plate will be used to harvest genomic DNA and perform the CEL-I assay, and one plate will be used as a working plate.

c. CEL-I Assay Procedure using DNA harvested from a 96-well plate

i. PCR amplify the genomic DNA. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Roche Expand High Fidelity^{PLUS} PCR System. Optimization of the conditions may be necessary if another polymerase is used.
Note: In addition to the transfected samples, it is recommended to include a control reaction using the included control genomic DNA (CDZFN).

Reagent	Volume
Water, PCR Reagent	Adjustable
5× PCR buffer	4 µl
dNTPs (10 mM)	0.4 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.2 µl
ZFN Primer F (25 µM)	0.4 µl
ZFN Primer R (25 µM)	0.4 µl
Genomic DNA (~2 µl if harvested as previously described for 96-well)	200 ng
Total volume	20 µl

Note: Volumes are small and should be used to make a master mix for a larger number of samples.

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30 cycles
Annealing	55 °C	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

ii. Take 10 µl of PCR reaction from each well and use the following program on a thermocycler:
95 °C, 10 minutes
95 °C to 85 °C, –2 °C/second
85 °C to 25 °C, –0.1 °C/ second
4 °C, indefinitely
Note: This step can also be performed without a thermocycler.
For specific instructions, see the Transgenomic User Guide

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for the Transgenomic SURVEYOR Mutation Detection Kit for Standard Gel Electrophoresis.

iii. Add 1 µl of enhancer and 1 µl of Nuclease S (from Transgenomic Catalog Number 706025) to each reaction and incubate at 42 °C for 40 minutes.
Note: A master mix of enhancer and nuclease S can be made just prior to digest. Do not allow enhancer and nuclease S to incubate together for extended periods prior to digest.

iv. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058).

Troubleshooting Guide

Problem	Cause	Solution
Genomic Control DNA (CDZFN) amplifies, but no amplification from transfected samples.	Quality of genomic DNA preparation is poor	Use a high quality genomic DNA isolation kit.
	Quantity of template	Make sure DNA concentration is measured accurately and use 200 ng of input template DNA.
The Genomic Control DNA did not amplify.	Most likely, the DNA polymerase used is not suitable for the amplification.	Try a different DNA polymerase. We highly suggest using the recommended polymerase and PCR conditions.
No CEL-I signal detected in your cell type.	Transfection efficiency is too low.	Optimize the transfection procedure to increase the efficiency, >50% is preferred.
	RNA integrity	Follow all proper procedures on handling RNA. To make sure the mRNAs are not degraded due to improper storage, check RNA integrity on a gel.
	The cells used are at a high passage number.	Low passage cells should be used. Low passage is generally considered less than 20 passages.
	Cell-to-cell variation in ZFN expression	Perform anti-FLAG Western blot analysis to assess ZFN expression.
No CEL-I PCR product at the single cell clone level from 96-well plate.	DNA is not pure.	Use a 96-well genomic DNA purification kit to yield higher quality DNA. The genomic DNA method stated in the Genomic DNA harvesting protocol for a 96-well plate is a quick method for extracting DNA, but it does not include any DNA purification steps. It is possible that unpurified DNA may make PCR amplification difficult.

CompoZr ZFN mRNA Production

The following protocols are intended for CompoZr ZFN users to produce additional ZFN mRNA in large quantity (60-80 µg) from a CompoZr ZFN plasmid construct provided by Sigma-Aldrich. Before the ZFN mRNA production, users first need to transform each of the two paired-ZFN plasmid constructs into an *E. coli.* strain and perform a midi or maxi scale plasmid purification for each construct. Purified plasmid is then digested into a linear form with XbaI and purified by phenol/chloroform extraction to generate a high quality DNA template for *in vitro* transcription.

Capped ZFN mRNA is produced from linearized plasmid DNA template by *in vitro* transcription with a MessageMAX™ T7 ARCA-Capped Message Transcription Kit. A poly(A) tail is then added to each ZFN mRNA by polyadenylation with a Poly(A) Polymerase Tailing Kit. Poly(A) tailed ZFN mRNA is then purified by spin column with a MEGAclear™ Kit. Finally, the two ZFN mRNAs are combined in equal amounts for use in gene knockout or target integration experiments.

Caution must be taken to avoid RNase contamination during ZFN mRNA preparation, especially during the RNA elution and post-elution handling steps. The work area and the pipette set must be free of RNases. Use RNase*ZAP* to decontaminate the work area and the pipette set if necessary. Use RNase-free pipette tips, preferably those with an aerosol barrier. Always wear gloves and change them often. Keep reagent vials and sample tubes closed when not in use.

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Reagents Required but Not Provided

XbaI (New England Biolabs, Catalog Number R0145S)

Phenol/Chloroform/Isoamyl Alcohol (Sigma-Aldrich, Catalog Number P2069)

3 M Sodium Acetate Buffer Solution (Sigma-Aldrich, Catalog Number S7899)

100% Ethanol (Sigma-Aldrich, Catalog Number 459884)

MessageMAX T7 ARCA-Capped Message Transcription Kit (Epicentre, Catalog Number MMA60710)

Poly(A) Polymerase Tailing Kit (Epicentre, Catalog Number PAP5104H)

ScriptGuard™ RNase Inhibitor (Epicentre, Catalog Number SRI6320K)

MEGAclear Kit (Ambion, Catalog Number AM1098)

Reagents Recommended but Not Provided

GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, Catalog Number NA0400)

RNase-free Water (Sigma-Aldrich, Catalog Number 95284)

RNase*ZAP* (Sigma-Aldrich, Catalog Number R2020)

Agilent RNA 6000 Nano Reagent Part 1and 2 (Agilent, Catalog Number 5067-1511)

Agilent 2100 Bioanalyzer (Agilent, Catalog Number G2938C)

a. DNA Template Preparation

i. Plasmid purification
   a. Transform each of the two paired-ZFN plasmid constructs provided by Sigma-Aldrich into an *E. coli* strain using kanamycin at 25 µg/ml for selection.
   b. Prepare a liquid culture for each plasmid construct from an isolated colony. LB is the preferred medium. Supplement the medium with kanamycin at 25 µg/ml. Perform a midi or maxi scale plasmid purification. It is highly recommended to use an endotoxin-free plasmid purification kit, such as GenElute HP Endotoxin-Free Plasmid Maxiprep Kit, to ensure low levels of endotoxins and residual RNase in purified plasmid preparations.

ii. Restriction enzyme digestion and post-digestion purification
   a. Set up a restriction enzyme digestion for each of the two plasmid constructs in a 1.5-ml microcentrifuge tube according the table below.

Plasmid DNA	20 µg
10X Buffer 4 (NEB)	10 µl
100X BSA	1 µl
XbaI (20 U/ µl)	8 µl
Sterile distilled water	Bring to total 100 µl
Total reaction volume	100 µl

   Incubate at 37 °C for 1-2 hours.

   b. Add 100 µl of phenol/chloroform/isoamyl alcohol (the bottom layer) to each digestion and vortex vigorously for at least 30 seconds. *Note: When using Sigma's phenol/chloroform/isoamyl alcohol, add the equilibration buffer into the phenol/chloroform/isoamyl alcohol and mix thoroughly and place the bottle in a refrigerator for at least 4 hours to separate the phases before use.*
   c. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature.
   d. Use a P-100 pipette and carefully transfer 50 µl of the supernatant into a clean 1.5-ml microcentrifuge tube. *Note: Place the pipette tip about half way into the supernatant layer and slowly aspirate the aqueous phase into the tip. Do not touch the inter-phase. If desired, up to 80 µl of the supernatant may be recovered. If more than 50 µl of the supernatant is recovered, increase the volume of 3 M sodium acetate and 100% ethanol proportionally in the next step; also increase the volume of RNase-free water proportionally to resuspend the DNA pellet.*
   e. Add 5 µl of 3 M sodium acetate solution and mix briefly. Add 150 µl of 100% ethanol and mix thoroughly to precipitate DNA.
   f. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature. Carefully pipette off the liquid. *Note: Place the tubes in a fixed orientation in the centrifuge, such as with the cap hinge outwards and check for the white pellet after centrifugation.* Always remove the liquid from the side opposite the pellet. Keep the same orientation in the subsequent centrifugation steps.
   g. Add 150 µl of 70% ethanol to wash the pellet. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature. Carefully remove all the liquid. *Note: If the pellet is too loose for all the liquid to be removed, centrifuge the tube again for 2 minutes before removing the remaining liquid.*
   h. Air-dry the pellet for 5 minutes and then resuspend in 8 µl of RNase/DNase free water. Vortex the tube to resuspend and then centrifuge briefly to collect the liquid. *Note: you may proceed immediately to in vitro transcription or store the sample at -20 °C for later use.*

iii. In Vitro Transcription with MessageMAX T7 ARCA-Capped Message Transcription Kit
   a. Set up a restriction enzyme digestion for each of the two plasmid constructs in a 1.5-ml microcentrifuge tube according the table below.
   b. Thaw RNase-free Water, 10X Transcription Buffer, ARCA Cap/NTP PreMix, 100 mM DTT, and plasmid DNA template, and centrifuge the tubes briefly. Warm these reagents to room temperature before assembling the reactions.
   c. Set up an *in vitro* transcription reaction for each of the two plasmid constructs. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature in the order given below:

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RNase-free Water	10 µl
Plasmid DNA template (~1 µg/µl)	2 µl
10X Transcription Buffer	4 µl
ARCA Cap/NTP PreMix	16 µl
100 mM DTT	4 µl
MessageMAX T7 Enzyme Solution	4 µl
Total reaction volume	40 µl

d. Incubate at 37 °C for 30 minutes. *Note: Extending the incubation time to 1 hour may increase the RNA yield in some instances.*

e. At the end of the *in vitro* transcription incubation, centrifuge the tube briefly and add 2 µl of DNase I. Mix gently and centrifuge briefly. *Note: Do not vortex the DNase I or the DNase I digestion.*

f. Incubate the DNase I digestion at 37 °C for 15 minutes. *Note: At the end of the DNase digestion, you may immediately proceed to the poly(A) tailing reaction or store the reaction at -20 °C overnight before proceeding to poly(A) tailing.*

b. Poly(A) Tailing with Poly(A) Polymerase Tailing Kit

i. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature according to the order given below:

RNase-free Water	109 µl
10X Reaction Buffer	20 µl
10 mM ATP	20 µl
ScriptGuard RNase Inhibitor (40 U/µl)	5 µl
In vitro transcription reaction	42 µl
Poly(A) Polymerase	4 µl
Total volume	200 µl

ii. Incubate the reaction at 37 °C for 1 hour. *Note: At the end of the poly(A) tailing reaction, you may proceed immediately to RNA purification or store the reaction at -20 °C overnight before purification.*

c. RNA Purification with MEGAClear Kit

i. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature according to the order given below:

ii. Add 700 µl of Binding Solution Concentrate to each poly(A) tailing reaction (200 µl) and mix thoroughly by vortex.

iii. Add 500 µl of 100% ethanol and mix thoroughly by vortex.

iv. Insert a Filter Cartridge into a Collection Tube and add 700 µl of the RNA sample into the Filter Cartridge.

v. Centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube. *Note: Centrifugation in this and all subsequent steps is performed at room temperature.*

vi. Add the remaining RNA sample into the same Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.

vii. Add 500 µl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.

viii. Add another 500 µl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.

ix. Dry the Filter Cartridge by centrifugation at ~16,000 xg for 1 minute.

x. Transfer the Filter Cartridge into a new Collection Tube. Add 50 µl of Elution Solution directly onto the center of the filter inside the Filter Cartridge. Close the cap of the tube immediately.

xi. Heat the tube at 65 °C for 10 minutes on a clean heat block or in a clean water bath.

xii. Centrifuge the tube at ~16,000 xg for 1 minute to elute RNA.

d. Determination of RNA quantity and quality, and RNA pooling

i. Combine 1 µl of eluted RNA sample with 99 µl of TE buffer to make a 100X dilution.

ii. Measure the RNA concentration and the A_{260}/A_{280} ratio of the diluted sample with a spectrophotometer or a NanoDrop instrument. When a spectrophotometer is used, RNA concentration and yield can be calculated as follows:
RNA concentration: A_{260} x dilution factor x 40 = µg/ml
RNA yield: 0.05 ml x RNA concentration (µg/ml) = µg
RNA yield should be ≥ 40 µg per transcription reaction. A_{260}/A_{280} should be ≥ 2.2.

iii. To assess RNA quality, prepare a 10-µl dilution aliquot at 80 ng/µl in a clean 1.5 ml microcentrifuge tube. Heat the aliquot at 65 °C for 5 minutes to denature, and run 1 µl of the sample on an Agilent bioanalyzer using the mRNA Nano Assay. The full length ZFN mRNA band should fall between the 1,000 and 2,000 nt markers. Poly(A) tailed CompoZr ZFN mRNAs are between 1,200 and 1,500 nt in length, dependent on how many zinc fingers the construct contains. Alternatively, RNA quality can be evaluated on agarose gel along with appropriate RNA markers as reference.

iv. Combine the two paired-ZFN mRNAs in an RNase-free tube in a concentration of 400 µg/ml each (standard formulation) or in a higher concentration if desired, following the examples below:
Example 1: Standard formulation (400 µg/ml each)

ZFN mRNA #1 (1400 µg/ml)	29 µl (40 µg)
ZFN mRNA #2 (1500 µg/ml)	27 µl (40 µg)
RNase-free Water	**44 µl**
Total volume	100 µl

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Example 2: Higher concentration formulation (700 µg/ml each)

ZFN mRNA #1 (1400 µg/ml)	50 µl (70 µg)
ZFN mRNA #2 (1500 µg/ml)	47 µl (70 µg)
RNase-free Water	**3 µl**
Total volume	100 µl

The combined ZFN mRNA is now ready for use. For storage, keep the combined ZFN mRNA at –20 °C for short-term storage (less than 6 months) or at –80 °C for long-term storage (longer than 6 months).

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4. Santiago, Y. *et al*. Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. *Proc Natl Acad Sci* U S A **105**, 5809-5814 (2008).
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For the most up to date reference list, please visit our website

FAQs

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